# Genetic diversity of citrus bacterial canker pathogens preserved in herbarium specimens


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**Citrus bacterial canker (CBC) caused by *Xanthomonas axonopodis* pv. *citri* (Xac) was first documented in India and Java in the mid 19th century. Since that time, the known distribution of the disease has steadily increased. Concurrent with the dispersion of the pathogen, the diversity of described strains continues to increase, with novel strains appearing in Saudi Arabia, Iran, and Florida in the last decade. Herbarium specimens of infected plants provide an historical record documenting both the geographic distribution and genetic diversity of the pathogen in the past. However, no method was available to assess the genetic diversity within these herbarium samples. We have developed a method, insertion event scanning (IES), and applied the method to characterize the diversity present within CBC populations documented as herbarium specimens over the past century. IES is based on the specific amplification of junction fragments that define insertion events. The potential for IES in current forensic applications is demonstrated by finding an exact match of pathogen genotypes preserved in herbarium specimens from Japan and Florida, demonstrating the source of the original outbreak of citrus canker in Florida in 1911. IES is a very sensitive technique for differentiating bacterial strains and can be applied to any of the several hundred bacteria for which full genomic sequence data are available.**



bacterial diversity | forensic pathology | *Xanthomonas*


Citrus bacterial canker (CBC) disease, caused by *Xanthomonas smithii* subsp. citri (1) = *Xanthomonas axonopodis* pv citri (2) is one of the most vexing problems of citriculture (3). The pathogen is spread by wind-driven rain (4, 5), and infection is facilitated, although the pathogen is not transmitted (6), by the citrus leaf miner *Phylocnictus citrella*. Symptoms include very distinctive raised hyperplastic lesions on leaves, stems, and fruit. These lesions weaken the tree, induce premature fruit drop, and render fruit unmarketable (7). All varieties of citrus are susceptible, and chemical controls are expensive and ineffective. Recurring outbreaks of CBC in Florida (1911, 1986, 1995, and subsequently) and Australia have therefore triggered vigorous and expensive eradication campaigns (4, 7, 8). The disease, which is endemic in China, Japan, southern Asia, and Oceania (3) is also endemic in Brazil since the 1950s, where it is managed by extensive survey programs to discover and eradicate infection foci in orange groves. Eradication campaigns mounted in response to a series of independent introductions of the pathogen into Florida since 1986 ended in January of 2006 when the United States Department of Agriculture and State of Florida declared the pathogen to be endemic in the state.

The United States Department of Agriculture Agricultural Research Service maintains a collection of 741 herbarium specimens of citrus with symptoms typical of CBC, including specimens from the first CBC outbreak in the United States, and numerous accessions originally from the Imperial Chinese and the Imperial Japanese herbaria and other collections, all of which substantially predate WWII (Fig. 1) (9, 10). Herbarium specimens are an invaluable resource for studies on origins, evolution, and phylogenetics of plants (11) and of their pathogens (12), and we wanted to study the relationships among the bacteria preserved in these specimens, using PCR. However, DNA in herbarium specimens is often degraded (13), and citrus plant materials contain compounds that inhibit the PCR (14). To overcome these problems, we have developed an efficient and reproducible protocol for the extraction of DNA from herbarium specimens with symptoms of CBC. DNA extracted from herbarium samples with this method was suitable for the amplification of specific products up to 542 bp long (15).

Various methods have been used to study genetic relationships among modern strains of *Xanthomonas axonopodis* pv. *citri*, starting with purified DNA from bacteria grown in axenic culture (16–18). However, such approaches cannot be used for herbarium specimens, because it is impossible to recover live bacteria or intact DNA from the herbarium samples. Thus, any analysis of such samples is necessarily an analysis of the DNA fragments left behind by the population of bacteria initially present in the herbarium specimens. The full genomic sequence of strain Xac306 of *X. axonopodis* pv. *citri* is available (19), and it is rich in transposable elements (TEs), most of which are located near strain-specific genes, in regions with altered codon usage and G+C content. We decided to develop a comprehensive set of primers targeted at these sites of TE insertions and use them to test the bacterial genomes present in the herbarium samples for the presence or absence of each specific insertion event.

Others have developed and successfully used a technique called TE display for studying population genetics of maize (20) and *Anopheles gambiae* (21). TE display allows the simultaneous discovery, in an autoradiogram of a DNA sequencing gel, of the insertion sites of hundreds of miniature inverted-repeat TEs present in maize or mosquito genomes. TE display was not ideal for our analyses because our target DNA was not intact, the specimens were not axenic, and the combinations of random and TE-specific primers used in TE display might result in spurious amplification products. Our method, which we call insertion event scanning (IES), uses a comprehensive set of primer pairs that specifically target TE


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Abbreviations: CBC, citrus bacterial canker; IES, insertion event scanning; MGS, mean genetic similarity; TE, transposable element; UPGMA, unweighted pair-group method with arithmetic means.


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**Fig. 1.** Historic specimens of citrus canker infected plant material preserved in the herbarium. The upper specimen (trifoliate orange rootstock) was deposited by H. E. Stevens from Florida in 1914 (29); the lower specimen (lemon) was originally deposited in the Imperial Japanese Herbarium in Yokohama from Nagasaki, Japan in 1915 (22).

insertion events defined by the reference strain Xac306 by standard-format PCR. IES is simple to perform and does not require restriction endonuclease digestion of DNA, radioisotope or autoradiography, nested-PCR reamplification of primary amplification products, or DNA sequencing gels used for TE display.

The taxonomic nomenclature referring to these bacteria has changed several times over the century because the pathogen was first named *Pseudomonas citri* after its introduction into Florida in 1911 (22). The herbarium specimens may include plants infected by either *X. smithii* subsp. *citri* [syn. *X. axonopodis* pv. *citri*] or *X. smithii* subsp. *aurantifolii* [syn. *X. axonopodis* pv. *aurantifolii*]. Under either current taxonomic proposal the variation within this group of bacteria is at the infra specific level. The hyperplastic lesions produced on leaves, fruit and stems by "citri" and "aurantifolii" are very similar. The two taxons have historically been distinguished by host and geography. The host range of aurantifolii is limited to lemons and limes in South America, whereas the host range of citri typically includes all citrus, worldwide. The taxons can also be distinguished by various DNA based tests (2) including total DNA::DNA hybridization analysis (1). All specimens were submitted to the herbarium as "citrus canker" specimens. Therefore, we refer to the bacteria in the herbarium specimens with the generic label "CBC."

## Results

We selected a total of 90 herbarium specimens with clear symptoms of CBC from 20 citrus and related species, originally obtained from 37 countries or island atolls. DNA was extracted from these samples and subjected to PCR-based amplification and analysis by IES. We designed three-primer sets for each of 53 TE insertion sites in the chromosome and the two plasmids of strain Xac306 (19). Each primer set has one primer specific to an end of a TE and another specific to the genomic region of insertion, so that each primer pair directs the amplification of a single amplicon, specific for a particular insertion event in the Xac306 genome. After comparing the three-primer sets for each TE insertion site, we selected the most robust primer pair for each insertion site. This set of primers directed the amplification of 53 unique products with an average size of 135 bp [see supporting information (SI) Table 2]. Amplifications were performed twice, using DNA extracted from CBC lesions from the 90 herbarium specimens and from six modern CBC strains as templates.

The reference strain Xac306 produced all 53 amplicons when tested by PCR, as expected. None of the 53 primer sets directed the amplification of products from all 90 herbarium samples. The range of the number of amplification products produced by the herbarium specimens (0–27) was consistent with that from contemporary bacterial cultures grown *in vitro* (2–45), showing no effect of template DNA source on PCR amplification. There was also no significant correlation between the number of TE insertion events detected as amplicons and the age of the herbarium specimens. Fifteen of the 90 herbarium extracts tested did not produce amplicons with any of the 53 primer pairs tested. Therefore, as a control for DNA extract quality, we used quantitative PCR directed at the citrus cytochrome oxidase gene (23), and the $C_t$ values obtained confirmed that all CBC-herbarium extracts contained DNA that supported amplification (Table 1). Finally CBC-herbarium extracts were tested by standard format PCR with primers Xac2/Xac3 (24) and J-pth1/J-pth2 (16, 25). These primers produce products of 222 and 197 bp, respectively, with all strains of Xac (26). All extracts produced the expected amplification products of Xac2/Xac3 and J-pth1/J-pth2, including the 15 CBC herbarium specimen extracts that did not produce any amplification products with the 53 IES primer pairs (Table 1). We sequenced representative DNA amplicons obtained from each TE insertion site in both the forward and reverse directions to confirm the specificity of the amplification reaction.

An insertion of ISXac3 in gene Xac 1921 was the most widely prevalent insertion event in our collection, present in 46 of the 90 herbarium specimens and five strains from pure culture (see below). We sequenced all PCR amplicons obtained with this primer pair. Analysis of the sequence data from this insertion sequence junction site shows that nearly all amplicons obtained from herbarium samples have the same DNA sequence of 122 bp, and this sequence is shared with modern Xac strains representing the current known diversity of the species. The three exceptions were amplicons that had either one or two single

**Table 1. Number of amplicons produced by IES, Taqman-based PCR assay for plant mitochondrial cytochrome oxidase gene and standard format PCR results, using primer sets of Xac2/Xac3 and J-pth1/J-pth2 directed at *X. axonopodis pv. citri* in extracts from herbarium specimens**

| Sample description | PCR amplicons from 53 IES sites | Xac2/Xac3 | J-pth2/J-pth3 | COX, $C_t$ |
|---|---|---|---|---|
| Argentina 1964 *Citrus sinensis* fruit | 0 | + | + | 25.00 |
| Kenya 1942 *C. aurantifolia* fruit | 0 | + | + | 27.66 |
| Japan 1952 *C. aurantium* fruit | 0 | + | + | 23.82 |
| Philippines 1919 *C. hystrix* leaves | 0 | + | + | 25.04 |
| South Africa 1942 *C. limonia* fruit | 0 | + | + | 20.62 |
| New Guinea 1954 *C. limon* fruit | 0 | + | + | 30.17 |
| Japan 1967 *C. unshui* fruit | 0 | + | + | 25.15 |
| Philippines 1915 *C. webberii* leaves | 0 | + | + | 20.81 |
| Philippines 1919 *C. grandis* leaves | 0 | + | + | 25.61 |
| Philippines 1915 *C. sinensis* leaves | 0 | + | + | 23.25 |
| Wake 1953 *C. sinensis* fruit | 0 | + | + | 25.23 |
| Ceylon 1938 *C. sinensis* fruit | 0 | + | + | 34.18 |
| Japan 1914 *C. grandis* leaves | 1 | + | + | 23.44 |
| Japan 1950 *C. limon* fruit | 3 | + | + | 25.10 |
| China 1919 (GX) *C. grandis* leaves | 2 | + | + | 23.64 |
| China 1919 (GD) *C. grandis* leaves | 14 | + | + | 23.98 |
| India 1938 *C. limon* leaves | 17 | + | + | 22.12 |
| Philippines 1921 *C. hystrix* leaves | 6 | + | + | 26.77 |
| Java 1919 *C. limonia* leaves | 6 | + | + | 26.70 |
| U.S. 1914 *Poncirus trifoliata* | 1 | + | + | 23.85 |
| Fresh healthy leaves of *C. sinensis* | n/a | − | − | 19.39 |
| Water | n/a | − | − | − |

COX, plant mitochondrial cytochrome oxidase gene (23).

nucleotide polymorphisms. Because of the absence of this insertion event from half of the samples tested, and the low level of sequence diversity in the amplicons produced, the sequence polymorphism data from this insertion sequence junction site did not provide useful information.

To validate IES for phylogenetic analysis, 53 individual insertion events were scored as present or absent in CBC DNA purified from extant strains. This set of extant strains was selected to include the bacterial diversity currently known in Xanthomonads that cause canker of citrus and included typical strains of *X. axonopodis* pv *citri* (Xac) from Japan and Brazil (XC62 and Xac306) and *X. axonopodis* pv *aurantifolii* (Xaa) from Brazil and Argentina (XC70 and XC94). The Xac strains have a wide host range within the Rutaceae and have a global distribution. In contrast, the Xaa strains have host ranges limited to lime or lemon do not occur outside of South America (3) and belong either to a separate pathovar or subspecies (1, 2). In addition to the typical strains of both Xac and Xaa, our set of extant reference strains included atypical strains of Xac recently isolated in Saudi Arabia (XC269) (27) and Florida (X01–3) (28). These strains, isolated from lime, have restricted host ranges and also differ from typical Xac serologically and in bacteriophage sensitivity. Two major clusters of strains were distinguished among these six extant CBC strains tested by IES, with a mean genetic similarity (MGS) of 32% (Fig. 2).

CBC samples from herbarium specimens were classified into four groups designated CBC1–CBC4 based on analysis of MGS to the reference strain Xac306 (Figs. 3 and 4). The IES data, which can be used to distinguish strains present in the herbarium specimens, can be combined with the collection date and geographical origin associated with the herbarium specimens to document the dispersal of the pathogen over time.

## Discussion

The quality of the DNA extracts is important in any PCR-based analysis, but it is especially critical in the analysis of rare or preserved specimens, such as those used in this study (15). In the present study, 15 of 90 CBC-herbarium extracts did not produce any amplicons from 53 insertion event specific primers, and other extracts produced relatively few amplification products. We therefore used three independent tests to confirm the quality of DNA in these extracts: amplification of the plant mitochondrial cytochrome oxidase gene by a Taqman-based assay (23) and amplication of Xac specific targets, using the well described primer pairs Xac1/Xac2 and J-pth1/J-pth2 (26). Both of these targets are plasmid borne and are present in all strains of Xac, and these primers comprise the standard PCR test for Xac in current use by the European Union (26). All CBC-herbarium extracts contained all three target DNAs (Table 1), confirming that the quality of the extracts was sufficient to support PCR and that they all contained Xac DNA. Thus, the results of the IES can be attributed with confidence to the presence or absence of the targeted insertion events and not to the lack of target DNA in the extracts or to poor quality extracts.

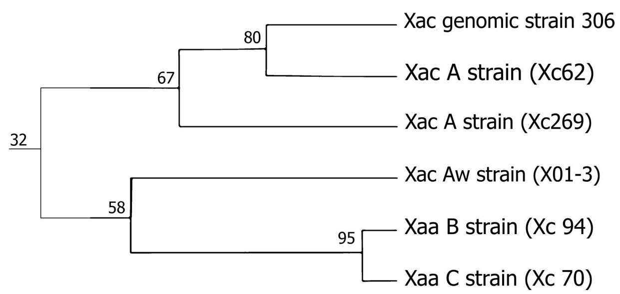


**Fig. 2.** Dendrogram (UPGMA) summarizing the MGS among extant CBC pathogens based on polymorphism data collected by IES. Scale reflects genetic distances among the bacterial strains detected in extant cultured specimens.

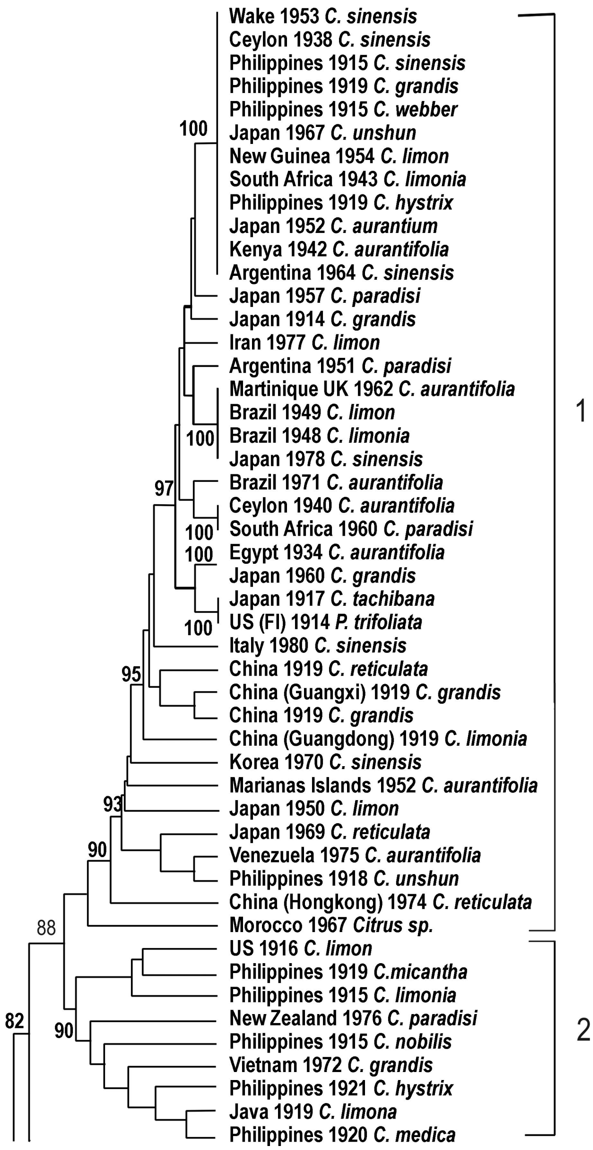


**Fig. 3.** Dendrogram (UPGMA) summarizing the MGS among CBC pathogens preserved in herbarium specimens based on polymorphism data collected by IES. Scale reflects genetic distances among the bacterial strains detected in historic herbarium specimens. CBC groups 1 and 2 are shown.

The ability of the 53 insertion site-specific amplification products to provide phylogenetic information was tested with DNA prepared from a set of extant strains of bacteria known to cause canker symptoms in citrus. A simple tree containing two major branches was produced from the set of six extant strains (Fig. 2). One of the branches included the typical Xac strains XC62 and Xac306 and the atypical Xac strain XC269. The other cluster contained three strains, the two typical Xaa strains, XC94 and XC70, and the atypical Xac strain X01–3. This tree is consistent with previous work with extant strains of this species, in which two clusters, designated Xac and Xaa, were described among extant strains with an MGS of 43 and 47% based on BOX- and ERIC-PCR data, respectively (16). BOX and ERIC are conserved and repeated sequence motifs in bacterial genomes that can be surveyed by PCR. In the present analysis, the MGS between XC269 (atypical Xac) and strains XC62 and Xac306 (typical Xac) is 67%, much lower than the MGS of 90% between the two typical Xac strains, consistent with the conclusion that XC269 represents a group of strains, designated A*, distinct

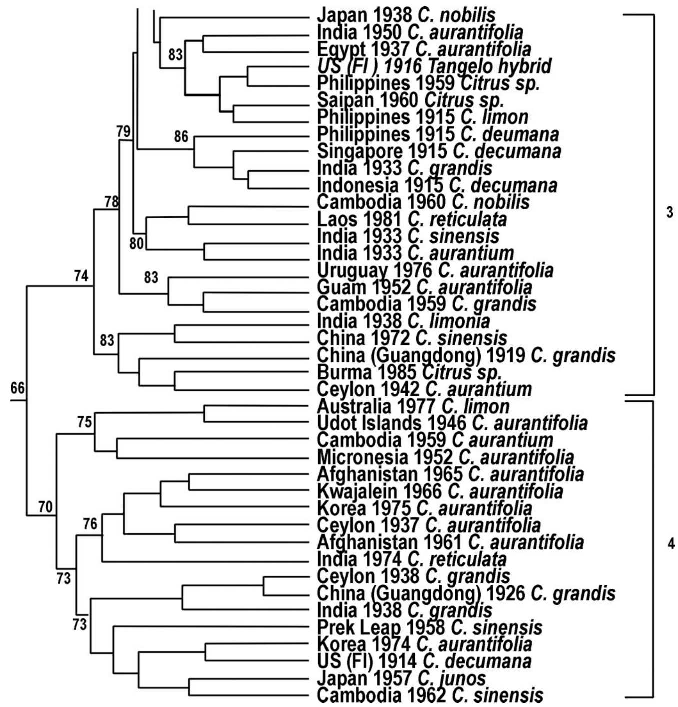


**Fig. 4.** Dendrogram (UPGMA) summarizing the MGS among CBC pathogens preserved in herbarium specimens based on polymorphism data collected by IES. Scale reflects genetic distances among the bacterial strains detected in historic herbarium specimens. CBC groups 3 and 4 are shown.

from other strains of Xac (27). Similarly, strains XC94 and XC70, representing Xaa, have low MGSs of 25 and 17%, respectively, to extant strains of Xac, as expected. Thus, our method for scoring genomes for the presence or absence of specific insertion events gave results consistent with previous extensive phylogenetic analyses (1, 2, 16).

Herbarium specimens were classified with this method into four groups designated CBC1–CBC4 based on the MGS to the reference strain Xac306 (Figs. 3 and 4). CBC1 is the group with the highest degree of relatedness within the group, with an MGS of 91% among the strains within the group. Within CBC1, many of the specimens have an MGS of 100% to an older Japanese or Philippine specimen (based on herbarium documentation). CBC2 includes several older samples from the Philippines among specimens from Indonesia, New Zealand, Vietnam, and Florida and has an MGS (as a group) of 88% to CBC1. CBC3 was more diverse, comprised of five subgroups designated a–e, related to one another with MGSs of 72.5% The oldest herbarium samples present in subgroups CBC3a and CBC3b were from the Philippines, and members of these groups were also found in specimens from Egypt, the United States, Indonesia, Singapore, and India. Similarly, the oldest specimens in the remaining subgroups (CBC3c–CBC3e) are from India with other specimens from Laos, Cambodia, Guam, Uruguay, Burma, Sri Lanka and China. CBC4 consisted of three subgroups with an MGS of 70%, each of which had more than one distinct evolutionary line of strains.

Although several countries, including Japan, the Philippines, China, Sri Lanka, and India, contributed herbarium samples with CBC belonging to two or three groups based on MGS, Florida was the only location, which had samples from all of the four CBC groups found in the herbarium specimens (Figs. 3 and 4). This genetic diversity is consistent with multiple introductions of Xac at the time of the first epidemics in Florida. The Florida sample on *Poncirus trifoliata* (1914) in the CBC1 group had a genetic similarity of 100% to the Japanese sample on *Citrus tachibana* (1919) (Fig. 3), consistent with the 90-year-old hypothesis that the first CBC in the U.S. was introduced from Japan on trifoliate orange rootstocks (29). Other CBC specimens from Florida have apparent affinity with herbarium specimens from the Philippines and Japan (Figs. 3 and 4). Citrus canker was reported to be widespread in both the Philippines and Japan by W. T. Swingle in 1915 (29), and the United States had extensive contact with both countries at that time.

We have documented at least four distinct genotypes present in herbarium specimens collected in Florida during the first eradication campaign directed at CBC in the early years of the 20th century. This previously unrecognized history of multiple introductions of CBC into Florida and the entire Gulf Coast of the U.S. in the early 20th century is entirely consistent with the analysis of the strains of CBC isolated during the current series of outbreaks of citrus canker in Florida, where two and three groups of strains were differentiated with ERIC and BOX elements, respectively, among 84 modern Xac strains isolated in Florida (16). Likewise, the substantial diversity of CBC found in the herbarium samples from Japan and China (CBC1, CBC3, and CBC4), the Philippines (CBC1, CBC2, and CBC4), and India (CBC3 and CBC4) is also consistent with analyses of contemporary disease outbreaks. Using ERIC- and BOX-PCR analysis of contemporary strains, two or three groups were also differentiated among the modern Xac strains isolated from India, China, and Japan (16).

Strains belonging to groups CBC1 and CBC2 were not found in herbarium samples from India, even though the center of origin of CBC is thought to be in Southeast Asia or India, on the basis of the presence of CBC symptoms in herbarium specimens from India (1827–1831) and Indonesia (Java) (1842–1844) at the Royal Botanical Gardens in Kew, England (10). Our results suggest that Japan was a center of diversity and may have been the primary center for the dissemination of CBC, with the Philippines and India acting as the secondary CBC dissemination centers. The center of origin of citrus canker still remains unknown, but it may not necessarily coincide with its center of diversity. This would be similar to the situation of the Irish potato famine pathogen, *Phytophthora infestans*, a conclusion also based on analysis of herbarium materials (12).

Our work demonstrates significant and previously unrecognized genetic diversity among citrus canker pathogens preserved in herbarium specimens, and helps us to understand the origins of past epidemics. For example, we demonstrate the genetic identity of populations of CBC found in herbarium specimens collected in Japan and in Florida at the time of the initial outbreak of CBC in Florida. This result confirms an old hypothesis concerning the source of CBC in the first U.S. outbreak of CBC, demonstrates the power of the IES technique, and demonstrates its potential usefulness in forensic plant pathology (30). The previously unrecognized genetic diversity among samples of CBC that we describe in the herbarium specimens should also alert plant quarantine agencies that many regional or local groups or subgroups of citrus canker pathogens documented in historic herbarium specimens may still persist in their centers of origin, diversity or proliferation. This would explain the continual emergence of novel strains of this pathogen, as has been observed repeatedly over the past decade, for example in Saudi Arabia (27), Florida (28), and Iran (31). Finally, we note that our work would not have been possible if earlier researchers had not deposited voucher specimens from their research in herbaria. Future researchers will benefit if contemporary researchers also contribute voucher specimens from their work to herbaria. In particular, the devastating citrus huanglongbing, or greening disease, has recently been found in both Brazil (32, 33) and Florida (23). Voucher specimens of these outbreaks should be deposited at herbaria for safekeeping and future study. The IES technique should be directly applicable to any of the several hundred bacterial species for which full genome sequence data are available.

## Materials and Methods

**Sampling and DNA Extraction.** A database was created for 741 herbarium samples that show symptoms of citrus canker. These samples were originally deposited from 37 countries and many citrus species and are housed at the U.S. National Fungus Collection of the United States Department of Agriculture Agricultural Research Service in Beltsville, MD (9, 10). The oldest sample from each country/host combination was selected for analysis. For each herbarium sample, 10 raised brown lesions with chlorotic margins were removed from leaves, bark, or fruit peels with a razor blade adjusted to 30 mg by cutting off or adding more boundary areas and placed in sterile lysing matrix A tubes (Qbiogene, Carlsbad, CA). To avoid cross contamination, each herbarium sample was opened, cut, and weighed with a new razor blade, new gloves, and new weighing paper in a room where no work with citrus canker bacteria had been conducted previously. DNA extraction with a sorbitol/cetyltrimethylammonium bromide buffer system was based on a comprehensive comparison of methods used for similar purposes (15). Extraction of DNA from *X. axonopodis* cultures was done by using the same procedure.

**PCRs for IES and DNA Sequencing.** PCRs contained 200 nm of each primer, 200 $\mu$M dNTPs, 2.5 mM $MgCl_2$, 0.5 unit of *Taq* polymerase (Roche, Indianapolis, IN), and 1 $\mu$l of 100-fold dilutions of DNA extracts and were performed in a final volume of 10 $\mu$l. The amplification program began with incubation at 94°C for 4 min, followed by 45 cycles of denaturation at 94°C for 30 s, annealing at 3°C below the consensus Tm of the primer pair (see SI Table 2) for 30 s, and extension at 72°C for 1 min, followed by a final extension cycle of 10 min at 72°C. PCR products were visualized by staining with ethidium bromide after electrophoresis through 1.5% agarose gels or through the E-gel system (Invitrogen, Carlsbad, CA). Amplifications were repeated one time.

PCR products were purified by using the Geneclean spin kit (Qbiogene) and inserted into the TOPO TA cloning vector pCR2.1. Chemically competent *Escherichia coli* TOP10 cells (Invitrogen) were transformed according to the manufacturer's instructions. Plasmid DNA was purified by using the RPM kit (Qbiogene). DNA sequencing was done at the DNA Sequencing Facility, University of Maryland, College Park, MD. Sequences were analyzed by BLASTn against the National Center for Biotechnology Information GenBank database.

**Standard Format PCR Assay for *X. axonopodis* pv. *citri*.** Reactions were carried out in a PTC-1000 instrument (MJ Scientific). Reaction conditions were 94°C for 2 min, followed by 35 cycles of 94°C for 10 s, 54°C (Xac2/Xac3) or 58°C (J-pth1/Jpth2) for 30 s, 72°C for 40 s, and finally one cycle of 72°C for 3 min. The reaction mixes contained the following: 1× reaction buffer, 2.5 mM MgCl2, 200 $\mu$l of dNTPs, 200 nM each primers, 1 unit platinum *Taq* (Invitrogen), and1 $\mu$l of DNA extract in a final volume of 25 $\mu$l. Primers Xac2/Xac3 and J-pth1/J-pth2, which produce amplicons of 227 and 197 nt, respectively (26), were used. Products were resolved in 1% agarose stained with ethidium bromide and viewed with UV light. A 100-bp ladder was used to estimate product sizes (Biomarker EXT, 50–2,000 bp; Bioventures, Murfreesboro, TN).

**Phylogenetic Analysis.** IES results were converted to binary form (0 = absence of specified amplicon; 1 = presence of specified amplicon). Relatedness among samples of CBC was analyzed by using the unweighted pair-group method with arithmetic

AGRICULTURAL SCIENCES

means (UPGMA) cluster analysis. Proportional difference was used to calculate the dendrogram based on the genome-wide IES data for the herbarium extracts. MGSs were calculated with NTSYS software, Version 2.1 (Exeter Software, Setauket, NY), using SIMQUAL with the Dice coefficient.

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